

Neuroepithelial circuit formed by innervation of sensory enteroendocrine cells

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Satiety and other core physiological functions are modulated by sensory signals arising from the surface of the gut. Luminal nutrients and bacteria stimulate epithelial biosensors called enteroendocrine cells. Despite being electrically excitable, enteroendocrine cells are generally thought to communicate indirectly with nerves through hormone secretion and not through direct cell-nerve contact. However, we recently uncovered in intestinal enteroendocrine cells a cytoplasmic process that we named neuropod. Here, we determined that neuropods provide a direct connection between enteroendocrine cells and neurons innervating the small intestine and colon. Using cell-specific transgenic mice to study neural circuits, we found that enteroendocrine cells have the necessary elements for neurotransmission, including expression of genes that encode pre-, post-, and transsynaptic proteins. This neuroepithelial circuit was reconstituted *in vitro* by coculturing single enteroendocrine cells with sensory neurons. We used a monosynaptic rabies virus to define the circuit's functional connectivity *in vivo* and determined that delivery of this neurotropic virus into the colon lumen resulted in the infection of mucosal nerves through enteroendocrine cells. This neuroepithelial circuit can serve as both a sensory conduit for food and gut microbes to interact with the nervous system and a portal for viruses to enter the enteric and central nervous systems.

Introduction

Satiety, food preference, and even mood behaviors are a few of the functions modulated by gut chemosensation (1). Ingested nutrients and bacterial by-products contacting the gut epithelium stimulate enteroendocrine cells (2). These are electrically excitable biosensors essential for normal life (3). The sensory mechanisms of enteroendocrine cells have recently been elucidated using transgenic fluorescence-reporter mice. For instance, cholecystokinin-GFP mice have enabled scientists to uncover how digested fats modulate metabolism. The mechanisms involve lipid stimulation of enteroendocrine cells through receptors such as GPR41 (4). Once stimulated, enteroendocrine cells secrete several neuropeptides, including cholecystokinin (CCK) and peptide YY (PYY), best known for their ability to induce satiety (5, 6).

Despite their recognized sensory function, how enteroendocrine cells relay sensory signals from the gut lumen onto nerves is poorly understood. Transmission has been regarded as paracrine, but not through direct enteroendocrine cell-nerve contact. Hormones secreted from enteroendocrine cells are thought to diffuse throughout the lamina propria until they reach the bloodstream or act on intrinsic sensory neurons or vagal afferent nerves (7, 8).

Although this is still a possibility, we recently uncovered a prominent cytoplasmic process in enteroendocrine cells of the small intestine and colon that we called neuropod (9, 10). This neuropod is escorted by enteric glia and elongates in the presence of neurotrophins; in addition, its tip almost invariably resembles a synaptic-like bouton, which suggests a physical connection to a nerve (9, 10).

Here, we studied such a possibility by using *Cck-GFP*, *Pyy-GFP*, and *Pyy-Cre* mice in combination with molecular tools for the study of neural circuits, such as monosynaptic rabies neurotracing and a Cre-dependent rabG mouse. We uncovered a new neuroepithelial circuit that has the potential to serve as a conduit between the lumen of the gut and the nervous system.

Results and Discussion

The contact with nerves. Because of their endocrine attribution, we first determined whether neuropods in enteroendocrine cells are associated with blood vessels. We revealed the vasculature of the small intestine and colon by perfusing *Pyy-GFP* transgenic mice with a buffer solution containing the lipophilic dye DiI. The technique is known as blood vessel painting (11). The results showed that blood vessels are found within 5.6 μm (SEM \pm 0.4, n = 3), but do not come into contact with enteroendocrine cells. We then immunolabeled the vessel-painted tissue with the panneuronal marker PGP 9.5 to determine the proximity of neuropods to nerve fibers innervating the mucosa. Nerves were observed penetrating the basal lamina and directly contacting the enteroendocrine cell neuropod (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI78361DS1). Figure 1, A–C, shows 3 examples of enteroendocrine cells contacting individual nerve fibers innervating the mucosa of the ileum and colon. The fre-

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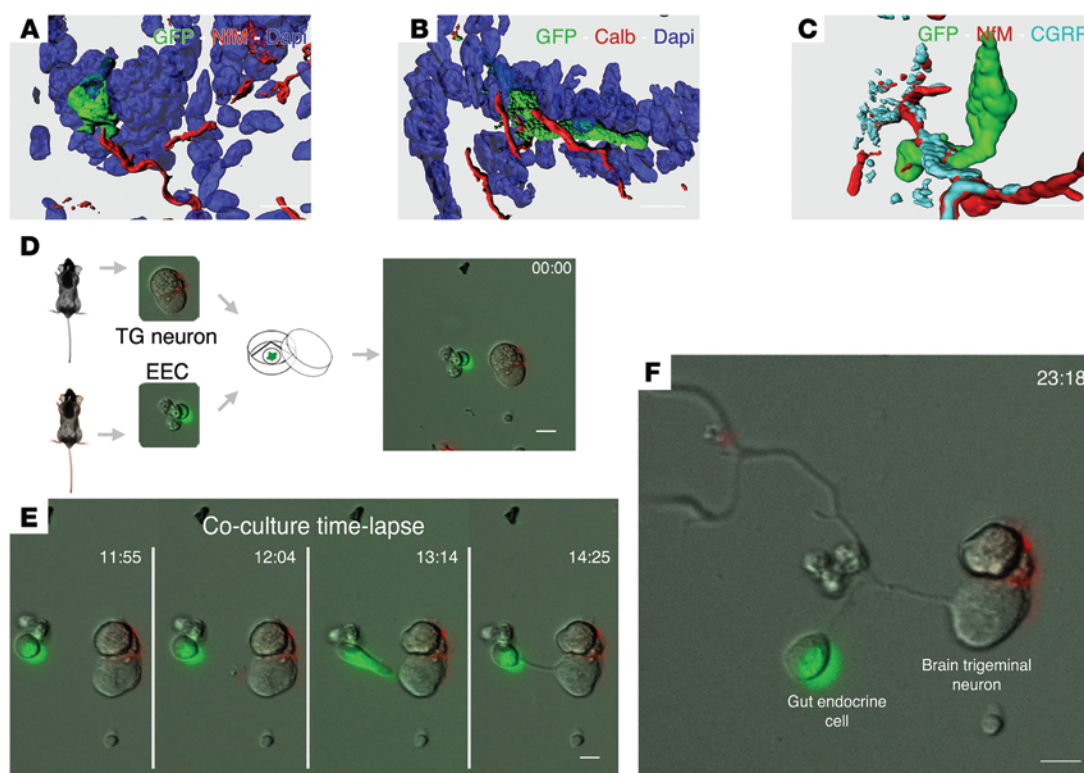


Figure 1. Enteroendocrine cells connect to sensory neurons in vivo and in vitro. (A–C) Confocal z-stacks were reconstructed using Imaris (Bitplane Inc.). *Cck-GFP* and *Pyy-GFP* enteroendocrine cells have a neuropod through which they contact to nerve fibers. (A) A neurofilament-medium (NfM) (red) nerve connects to an intestinal *Pyy-GFP* cell (green). (B) Calbindin (Calb) nerve innervates the neuropod of a colonic *Pyy-GFP* cell. (C) Colonic *Pyy-GFP* cell extends its neuropod to connect to neurofilament and CGRP nerves. (D) Coculture scheme of enteroendocrine cell and primary sensory neurons. EEC, enteroendocrine cell; TG, trigeminal neuron. (E) Time-lapse sequence showing how a single *Cck-GFP* enteroendocrine cell (green) connects to a sensory neuron (DiI-labeled, red) in vitro. Footage is presented in Supplemental Videos 1 and 2. (F) The enteroendocrine cell-neuron connection is stable at 23 minutes, 14 seconds, and cells remained connected for 88 hours until the end of the experiment. Time, hours:minutes. Scale bars: 10 μ m.

quency of contacts was conserved in both types of tissue, but varied depending on the neuronal marker used. For instance, in the colon, 67.3% (SEM \pm 2.7, n = 5) of *Pyy-GFP* enteroendocrine cells contacted PGP 9.5 nerves, 57.3% (SEM \pm 2.7, n = 5) contacted neurofilament-medium nerves, and 60.9% (SEM \pm 1.4, n = 5) contacted calbindin-positive nerves. Besides calbindin, some *Pyy-GFP* also contacted calcitonin gene-related peptide (CGRP) nerves, which have been described as markers of sensory neurons (12, 13). No enteroendocrine cells were seen contacting nerve fibers immunoreactive for vasoactive intestinal peptide, which has been used as a marker of motor neurons. These data show that PYY-secreting enteroendocrine cells of the ileum and colon are in contact with nerve fibers.

The connection to sensory neurons is recapitulated in vitro. To test the specificity of this connection, we developed a coculture method of enteroendocrine cells and sensory neurons (Figure 1D). Enteroendocrine cells were isolated and purified by FACS from the small intestine of *Cck-GFP* mice (14), and sensory neurons were enzymatically dissociated from the trigeminal or dorsal root ganglia of wild-type mice (15). Once isolated, enteroendocrine cells and sensory neurons were cocultured and imaged over days using a fluorescence incubator microscope (Figure 1D).

Figure 1, E and F, shows a representative time-lapse sequence of the events after plating single enteroendocrine cells and sensory neurons together. The complete event is documented in Supple-

mental Video 1. At 11 hours, 55 minutes, an enteroendocrine cell (*Cck-GFP*, green) is observed lying next to a neuron (lipophilic DiI, red). Next, the neuron extends a small neurite (arrowhead) toward the enteroendocrine cell (12 hours, 4 minutes), and the enteroendocrine cell responds by elongating a cytoplasmic process toward the neuron (13 hours, 14 minutes) that connects to the neuron's neurite (14 hours, 25 minutes). The sensory neuron continues extending a putative axon, while the enteroendocrine cell remains connected (23 hours, 18 minutes). In this example, the enteroendocrine cell and the neuron remained connected until the experiment was ended after 88 hours. An additional example is shown in Supplemental Video 2. In this case, a neuron clearly elongates an axon that connects with a distant enteroendocrine cell. The footage in both examples is unique because it contains information that can only be appreciated by observing the sequence in motion.

This kind of interaction was not evident in control experiments when enteroendocrine cells were cocultured with non-GFP epithelial cells or HEK immortal cells. Two days after plating, it was estimated that about 15% of all *Cck-GFP* cells were observed in contact with a neuron or a neuronal fiber. It is noteworthy that the viability of single *Cck-GFP* enteroendocrine cells in culture is low. One day after plating, only 5 *Cck-GFP* cells for every 1,000 remained alive ($0.50\% \pm 0.17\%$ at d1 to $0.10\% \pm 0.02\%$ at d6; average of 3 wells per replicate, n = 6). Nonetheless, the fact that the connectiv-

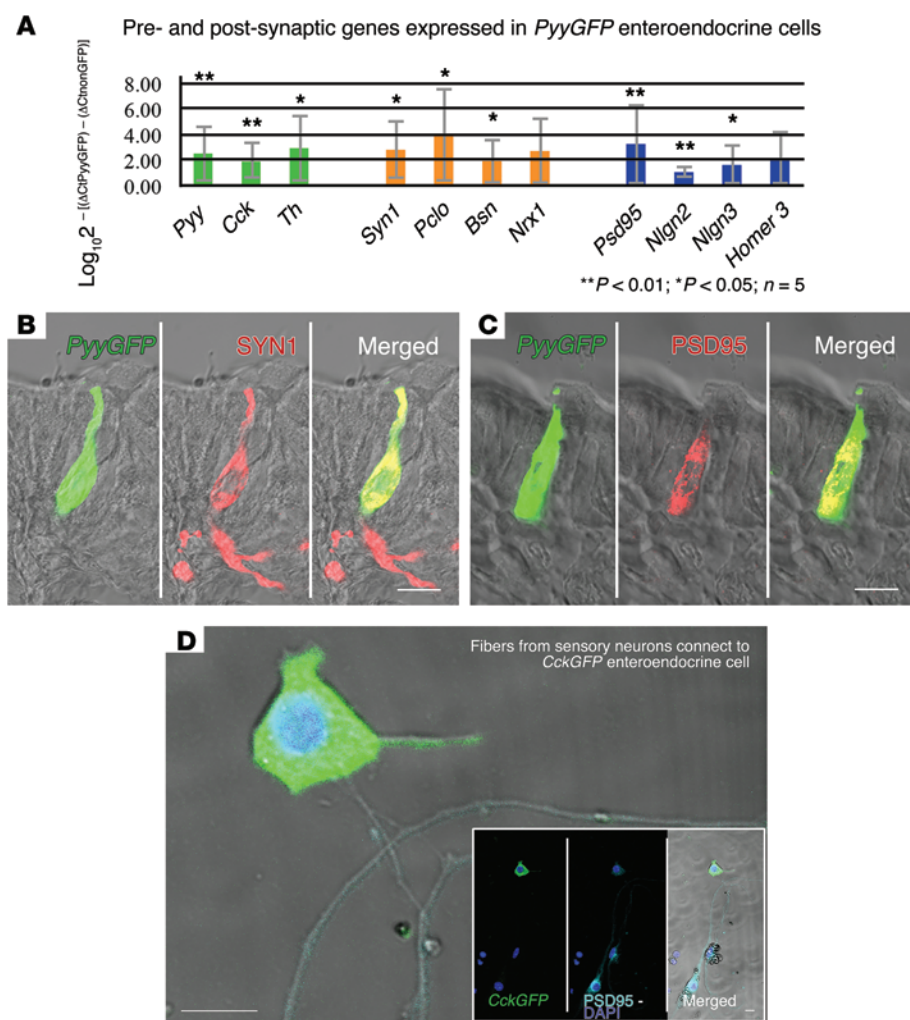


Figure 2. Enteroendocrine cells express pre- and postsynaptic proteins. (A) Gene expression analysis of FAC-sorted *Pyy-GFP* enteroendocrine cells shows their expression of presynaptic and postsynaptic proteins. Fold expression represents $\log_2 \left[\frac{(\Delta Ct \text{ } Pyy\text{-}GFP\text{-positive cells})}{(\Delta Ct \text{ } GFP\text{-negative epithelial cells})} \right]$ and error bars represent the SEM. Significant differences were separated by Student's *t* test using $2^{-(\Delta Ct)}$ values at $\alpha = 0.05$. (B) The presynaptic marker synapsin 1 (SYN1) immunoreacted with 96.4% (SEM \pm 0.5%) of *Pyy-GFP* cells. (C) 35.3% (SEM \pm 3.5%) of *Pyy-GFP* cells immunoreacted with the postsynaptic marker PSD95. (D) In vitro, *Cck-GFP* cells connecting to trigeminal neurons expressed the postsynaptic marker PSD95 (cyan). Blue, DAPI nuclear stain. Scale bars: 10 μ m.

ity is recapitulated in vitro is of great significance because it demonstrates the specific affinity between enteroendocrine cells and extrinsic sensory neurons in the absence of any other cell input.

A note on the enteroendocrine cells' life span. It has been proposed that because epithelial cells, including enteroendocrine cells, turn over every 5 days, the window for enteroendocrine cells to connect to neurons is restricted (7). However, some reports have suggested that enteroendocrine cells may live longer than epithelial enterocytes (16). We wondered whether *Pyy-GFP* enteroendocrine cells may live longer than 5 days and used BrdU pulse labeling in *Pyy-GFP* mice to determine their life spans compared with other intestinal epithelial cells. Although the initial number of labeled *Pyy-GFP* cells was low (6.3% \pm 1.2%; *n* = 3), there was evidence of labeled *Pyy-GFP* enteroendocrine cells 60 days after labeling, indicating that long-lived epithelial cells constitute a portion of the enteroendocrine cell population. Examples are presented in

Supplemental Figure 2. These data suggest that at least a population of enteroendocrine cells may have a comparable life span to that of other innervated biosensors, such as olfactory receptor neurons or taste cells (17, 18).

Synaptic features in enteroendocrine cells. Electron microscopy studies have revealed that, besides hormone-containing large dense-core vesicles, enteroendocrine cells also have small clear synaptic vesicles (19), suggesting that enteroendocrine cells have an essential organelle for neurotransmission. Thus, we considered that the enteroendocrine cell–neuron connection could have features of a synapse. We purified *Pyy-GFP* enteroendocrine cells by FACS and analyzed the gene expression encoding for defined proteins found in pre- and postsynapses by quantitative reverse-transcriptase PCR (qRT-PCR) (20). Indeed, compared with non-GFP intestinal epithelial cells, intestinal and colonic enteroendocrine cells expressed a number of genes encoding for presynaptic proteins, including: synapsin 1, piccolo, bassoon, MUNC13B, RIMS2, latrophilin 1, and transsynaptic neuroligin 2 (21). *Pyy-GFP* enteroendocrine cells also expressed DOPA decarboxylase and tyrosine hydroxylase — both essential enzymes in synthesis of the neurotransmitter dopamine (Figure 2A and Supplemental Table 1).

Pyy-GFP enteroendocrine cells also expressed a cohort of postsynaptic genes, including the transsynaptic neuroligins 2 and 3, homer 3, and postsynaptic density 95 (*Psd95*) (22).

Using immunohistochemistry, we found that, of all colonic *Pyy-GFP* enteroendocrine cells, 96.4% (SEM \pm 0.6) expressed the presynaptic marker synapsin 1 (Figure 2B) and 35.2% (SEM \pm 3.5) the postsynaptic marker PSD95 (Figure 2C). In vitro, enteroendocrine cells cocultured with neurons also expressed synaptic markers. Figure 2D shows an example of 2 separate fibers from neurons in culture that connect to a *Cck-GFP* enteroendocrine cell immunoreactive for PSD95. It is possible that, by comparing the expression to non-GFP cells, other synaptic genes may have been overlooked because the population of non-GFP epithelial cells also contains unsorted enteroendocrine cells that do not express PYY.

These data show that enteroendocrine cells have the necessary elements to engage in afferent and efferent synaptic transmission. Enteroendocrine cells are already thought to relay afferent signals from nutrients to underlying visceral nerves via hormones (23), but their expression of postsynaptic proteins raises the possibility of

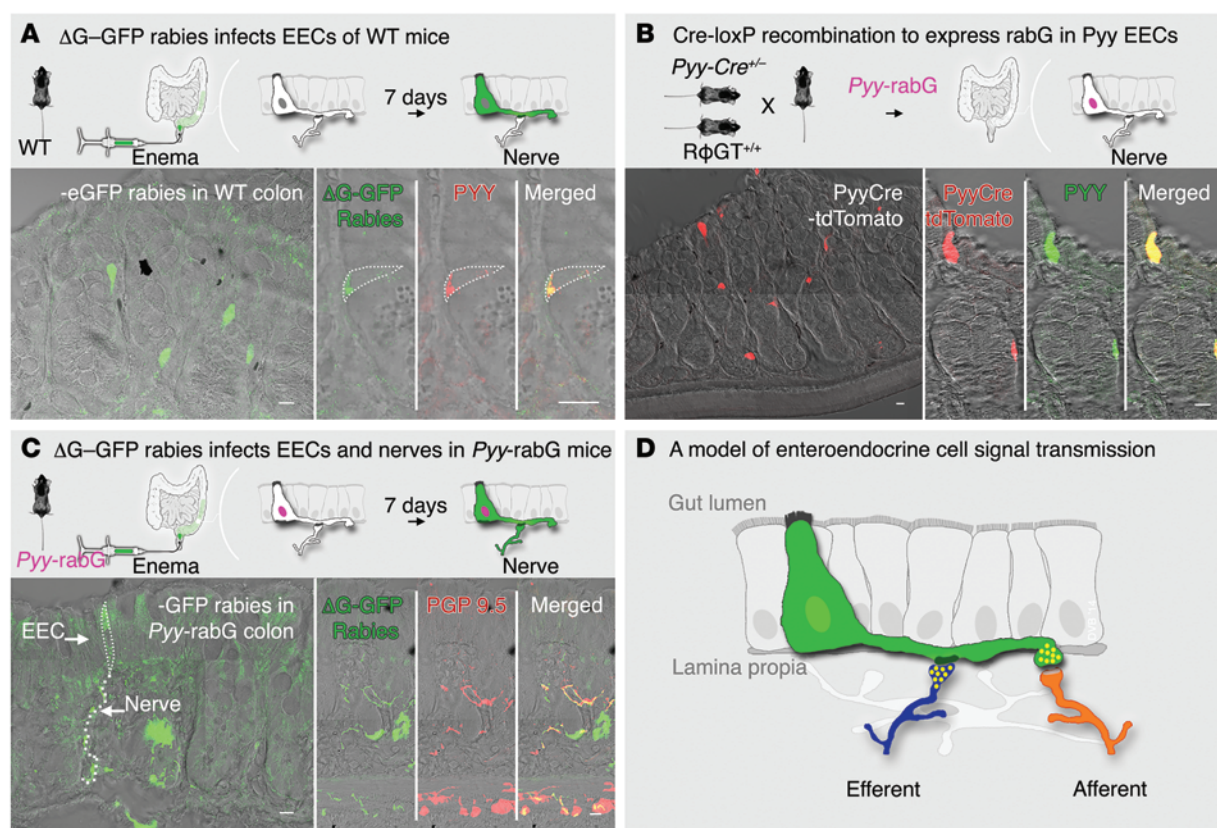


Figure 3. Enterοendoϋrine cell-neuron circuit revealed by monosynaptic rabies virus. (A) When delivered by enema into the lumen of the colon of wild-type mice, SAD Δ G-GFP (Δ G-GFP) rabies infects enteroendocrine cells, as confirmed by their PYY immunoreactivity. (B) To enable the monosynaptic rabies to spread, we engineered rabG glycoprotein in PYY-containing enteroendocrine cells by breeding *Pyy-Cre* with *R ϕ GT* mice. (C) When delivered into the colon of *Pyy-Cre-rabG* (*Pyy-rabG*) mice, SAD Δ G-GFP infected enteroendocrine cells (dotted lines) and underlying connecting nerve fibers. These immunoreacted with the neuronal marker PGP 9.5. (D) Model of afferent and efferent innervation of enteroendocrine cells. Scale bars: 10 μ m.

efferent neurotransmission. In other sensory epithelial cells, such as hair cells of the inner ear, the responsiveness to the mechanical stimulus of sound is toned down through efferent feedback (24). Likewise, efferent neurotransmission in enteroendocrine cells might serve to modulate the responsiveness of enteroendocrine cells to nutrients and bacterial by-products in the lumen of the intestine.

Rabies infection and neurocircuit connectivity. We sought to define this neural circuit in vivo. For this, we used an established neurotracing method based on a monosynaptic rabies virus. The virus is known as SAD Δ G-GFP rabies because its envelope glycoprotein rabG has been replaced with GFP (25). rabG is responsible for the spread of rabies from neuron to neuron through synapses. Thus, a monosynaptic neural circuit can be defined by expressing rabG into the target cell to allow the virus to jump 1 synapse and reveal the 2 connected neurons by fluorescence. Rabies has an affinity for neurons, and despite the neuronal features of enteroendocrine cells (9), it has been unknown whether rabies could infect enteroendocrine cells. We first tested to determine whether SAD Δ G-GFP rabies virus could infect enteroendocrine cells when delivered by enema into the lumen of the distal colon in P3 wild-type mice. The results showed that 7 days after infection, SAD Δ G-GFP rabies infected epithelial cells that closely resemble enteroendocrine cells (Figure 3A). Rabies virus has been shown to infect neurons through 3 molecular receptors; one of those is NCAM (26), which is expressed in *Pyy-*

GFP enteroendocrine cells (Supplemental Table 1). Immunoreactivity with a PYY antibody confirmed that some of these cells are PYY-secreting enteroendocrine cells (Figure 3A). No cells below the epithelium, including nerves, were infected by rabies (Figure 3A). These data indicate that SAD Δ G-GFP rabies infects enteroendocrine cells, but in the absence of rabG, cannot spread further.

We then engineered rabG in a subset of enteroendocrine cells to allow SAD Δ G-GFP rabies to travel 1 synapse. Using Cre-LoxP recombination, we bred *R ϕ GT* (27) and *Pyy-Cre* (28) mouse lines to develop a *Pyy-Cre-rabG* mouse. In this mouse, only PYY-secreting enteroendocrine cells express rabG. Then we delivered SAD Δ G-GFP by enema into the lumen of the distal colon of P3 *Pyy-Cre-rabG* mice. The results showed that 7 days after infection, SAD Δ G-GFP rabies infects enteroendocrine cells and, more importantly, their connecting neurons. The neuronal fibers can be clearly distinguished by fluorescence in the lamina propria of the distal colon. GFP fibers in the intestinal mucosa were confirmed as nerves by their colocalization with the neuronal marker PGP 9.5 (Figure 3C). Figure 3C shows an epithelial enteroendocrine-like cell (dotted lines) expressing GFP, as well as nerve fibers in the intestinal mucosa. Whether these are intrinsic or extrinsic neurons connecting to enteroendocrine cells remains to be characterized. Infection was observed in 4 out of 6 infected *Pyy-Cre-rabG* mice, and no infection of cells in the lamina propria was evident in the 6 wild-type

mice that served as controls. Because rabies virus spreads through synapses in a retrograde fashion, these data highlight that enteroendocrine cells are synaptically connected to efferent neurons in vivo.

Although paracrine transmission remains a possibility, we believe that the physical innervation of sensory enteroendocrine cells opens the following possibilities: (a) precise temporal transmission of sensory signals from the gut lumen; (b) real-time modulatory feedback onto enteroendocrine cells; (c) localized plasticity to sensory stimuli in the gut; (d) precise topographical representation of sensory signals from the gut; and (e) given the conditions, a potential physical path for viruses in the lumen of the gut to gain access to the peripheral or central nervous system.

Methods

The Supplemental Methods section contains procedures for cocultures, BrdU pulse labeling, rabies neurotracing, and imaging.

Study approval. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Duke University Medical Center.

Statistics. Statistical differences were determined using a 2-tailed *t* test analysis at $\alpha = 0.05$.

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- Critchley HD, Harrison NA. Visceral influences on brain and behavior. *Neuron*. 2013;77(4):624–638.
- Furness JB, Rivera LR, Cho HJ, Bravo DM, Callaghan B. The gut as a sensory organ. *Nat Rev Gastroenterol Hepatol*. 2013;10(12):729–740.
- Mellitzer G, et al. Loss of enteroendocrine cells in mice alters lipid absorption and glucose homeostasis and impairs postnatal survival. *J Clin Invest*. 2010;120(5):1708–1721.
- Samuel BS, et al. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *Proc Natl Acad Sci U S A*. 2008;105(43):16767–16772.
- Batterham RL, et al. Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature*. 2002;418(6898):650–654.
- Gibbs J, Young RC, Smith GP. Cholecystokinin elicits satiety in rats with open gastric fistulas. *Nature*. 1973;245(5424):323–325.
- Bertrand PP. The cornucopia of intestinal chemosensory transduction. *Front Neurosci*. 2009;3:48.
- Cummings DE, Overduin J. Gastrointestinal regulation of food intake. *J Clin Invest*. 2007;117(1):13–23.
- Bohórquez DV, Samsa LA, Roholt A, Medicetty S, Chandra R, Liddle RA. An enteroendocrine cell-enteric glia connection revealed by 3D electron microscopy. *PLoS One*. 2014;9(2):e89881.
- Bohórquez DV, Chandra R, Samsa LA, Vigna SR, Liddle RA. Characterization of basal pseudopod-like processes in ileal and colonic PYY cells. *J Mol Histol*. 2011;42(1):3–13.
- Li Y, Song Y, Zhao L, Gaidosh G, Laties AM, Wen R. Direct labeling and visualization of blood vessels with lipophilic carbocyanine dye DiI. *Nat Protoc*. 2008;3(11):1703–1708.
- McCoy ES, Taylor-Blake B, Street SE, Pribisko AL, Zheng J, Zylka MJ. Peptidergic CGRPalpha primary sensory neurons encode heat and itch and tonically suppress sensitivity to cold. *Neuron*. 2013;78(1):138–151.
- Song ZM, Brookes SJ, Costa M. All calbindin-immunoreactive myenteric neurons project to the mucosa of the guinea-pig small intestine. *Neurosci Lett*. 1994;180(2):219–222.
- Wang Y, et al. Amino acids stimulate cholecystokinin release through the Ca²⁺-sensing receptor. *Am J Physiol Gastrointest Liver Physiol*. 2011;300(4):G528–G537.
- Malin SA, Davis BM, Molliver DC. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nat Protoc*. 2007;2(1):152–160.
- Tsubouchi S, Leblond CP. Migration and turnover of entero-endocrine and caveolated cells in the epithelium of the descending colon, as shown by radioautography after continuous infusion of 3H-thymidine into mice. *Am J Anat*. 1979;156(4):431–451.
- Hamamichi R, Asano-Miyoshi M, Emori Y. Taste bud contains both short-lived and long-lived cell populations. *Neuroscience*. 2006;141(4):2129–2138.
- Hinds JW, Hinds PL, McNelly NA. An autoradiographic study of the mouse olfactory epithelium: evidence for long-lived receptors. *Anat Rec*. 1984;210(2):375–383.
- Nilsson O, Bilchik AJ, Goldenring JR, Ballantyne GH, Adrian TE, Modlin IM. Distribution and immunocytochemical colocalization of peptide YY and enteroglucagon in endocrine cells of the rabbit colon. *Endocrinology*. 1991;129(1):139–148.
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29(9):e45.
- Sudhof TC. The presynaptic active zone. *Neuron*. 2012;75(1):11–25.
- Chen X, et al. Organization of the core structure of the postsynaptic density. *Proc Natl Acad Sci U S A*. 2008;105(11):4453–4458.
- Okano-Matsumoto S, McRoberts JA, Tache Y, Adelson DW. Electrophysiological evidence for distinct vagal pathways mediating CCK-evoked motor effects in the proximal versus distal stomach. *J Physiol*. 2011;589(pt 2):371–393.
- Castellano-Munoz M, Israel SH, Hudspeth AJ. Efferent control of the electrical and mechanical properties of hair cells in the bullfrog's sacculus. *PLoS One*. 2010;5(10):e13777.
- Wall NR, Wickersham IR, Cetin A, De La Parra M, Callaway EM. Monosynaptic circuit tracing in vivo through Cre-dependent targeting and complementation of modified rabies virus. *Proc Natl Acad Sci U S A*. 2010;107(50):21848–21853.
- Thoulouze MI, Lafage M, Schachner M, Hartmann U, Cremer H, Lafon M. The neural cell adhesion molecule is a receptor for rabies virus. *J Virol*. 1998;72(9):7181–7190.
- Takato J, et al. New modules are added to vibrissa premotor circuitry with the emergence of exploratory whisking. *Neuron*. 2013;77(2):346–360.
- Schönhoff S, et al. Energy homeostasis and gastrointestinal endocrine differentiation do not require the anorectic hormone peptide YY. *Mol Cell Biol*. 2005;25(10):4189–4199.